REMARKS

With this amendment, Claims 10-17, 20 and 21 are pending. Claims 10-14 are cancelled without prejudice to subsequent revival. Claims 17, 20 and 21 are withdrawn by the Examiner as allegedly being outside the scope of the originally elected invention. Applicants respectfully traverse this withdrawal. Claims 15 and 16 are rejected. For convenience, the Examiner's rejections are addressed in the order presented in the December 18, 2002 Office Action.

I. Status of the Claims

Claims 15 and 16 are currently under examination and are rejected. Applicants also traverse the restriction requirement imposed by the Examiner and assert that claims 17, 20 and 21, which depend on claim 15, should also be under examination.

Claim 17 has been amended to recite that the candidate bioactive agent is a member of a library of candidate bioactive agents and the cell is a member of a plurality of cells. Support for this amendment is found, for example, at page 3, lines 22-24 and page 24 line 18 through page 25, line 7. This amendment adds no new matter.

Claim 20 has been amended to recite further comprising determining the acitivty of the R0101 protein in the presence of the candidate bioactive agent. Support for this amendment is found, for example, at page 3, lines 19-22. This amendment adds no new matter.

II. Election/Restriction

The Examiner alleges that claims 17, 20, and 21 are directed to inventions that are independent or distinct from the originally claimed invention, *i.e.*, Group I, and has thus restricted claims 17, 20, and 21 and withdrawn them from prosecution. Applicants respectfully traverse the restriction.

Elected Group I is directed to a method of screening for a bioactive agent that is capable of binding to cell cycle protein R0101 and is represented by claims 15 and 16, currently under examination. In order for claimed inventions to be independent, the inventions must not be connected in design, operation, or effect under the disclosure of the application under consideration. MPEP 802.01. In order for claimed inventions to be distinct, the inventions must not be patentable over each other. MPEP 802.01.

The Office Action asserts that claims 17, 20 and 21 are "outside the limitations of Claims 15 and 16" (page 2). Applicants point out that each of Claims 17, 20 and 21 depend from Claim 15, thereby comprising all of the limitations of this independent claim. Claim 15 describes a method comprising 2 steps; the "comprising" provides that any procedure having these two steps, regardless of how many other steps are involved, is encompassed by this claim. Therefore, claims 17, 20 and 21 cannot be outside the limitations of Claim 15.

The Office Action alleges that claim 17 is drawn to a method comprising steps that are not required to screening for a bioactive agent that binds to cell cycle protein R0101, essentially the subject matter of elected Group I. Applicants respectfully point out that claim 17, like claim 16, depends on claim 15 and is directed to the method of claim 15 done using multiple bioactive agents (*i.e.*, a library of bioactive agents) at the same time. Thus, claim 17 and claims 15 and 16 share design, operation, and effect and are not independent.

The Office Action alleges that claim 20 is outside the scope if the elected group and thus is separate and distinct from claims 15 and 16. Applicants respectfully point out that claim 20, like claim 16, depends on claim 15 and is directed to the method of method of claim 15 done with the additional step of determining the activity of R0101 in the presence of a candidate bioactive agent. One of the activities of the claimed cell cycle protein is binding to PCNA. (Specification at page 31, lines 11-15.) A functional binding relationship between R0101 and PCNA is a limitation of claim 15. Thus, the differences in design between the claims are negligible and claims 15 and 20 have the

same operation and effect, *i.e.*, determining binding of the bioactive agent to the cell cycle protein R0101.

The Office Action alleges that claim 21 is outside the scope if the elected group and thus is separate and distinct from claims 15 and 16. The Office Action further alleges that claim 21 reads on a method of making a composition *per se*. Applicants respectfully traverse and remind the Examiner that a dependent claim is construed to include all the limitations of the claim incorporated by reference into the dependent claim. Applicants respectfully point out that claim 21, like claim 16, depends on claim 15 and is thus also directed to a method for screening a bioactive agent capable of binding to the cell cycle protein R0101. There is no limitation in claim 21 to suggest that a method of making a composition is being claimed. Claim 21 is directed to the method of claim 15 done in the presence of PCNA. A functional binding relationship between R0101 and PCNA is a limitation of claim 15. Thus, the differences in design between the claims are negligible and claims 15 and 21 have the same operation and effect, *i.e.*, determining binding of the bioactive agent to the cell cycle protein R0101.

If Claim 15 is patentable, claims 17, 20 and 21 are necessarily also patentable. However, since claims 17, 20 and 21 are species of claim 15, claim 15 cannot be patentable over any of claims 17, 20 and 21. Furthermore, as discussed above, the examined and excluded claims are connected in connected in design, operation, and effect because they necessarily comprise identical steps. Claims 17, 20 and 21 are not independent and distinct from claim 15 and the exclusion of claims 17, 20 and 20 from prosecution in the present case is inconsistent with the rules set forth in MPEP 802.01. Therefore, Applicants respectfully request inclusion of claims 17, 20 and 21 in the prosecution of the present application.

III. Information Disclosure Statement

According to the Examiner, the original IDS filed on June 2, 2002 fails to comply with 37 C.F.R. 1.98(a)(2) because the statement and the appended references are

illegible. Applicants enclose new copies of the IDS and submitted references and respectfully request that the Examiner consider them.

IV. Rejection under 35 U.S.C. §101

Claims 15 and 16 are rejected under 35 U.S.C. §101 for allegedly lacking specific, substantial, or credible utility. Applicants respectfully traverse.

The essential basis of this rejection is the assertion that the protein R0101, as described in the present specification is "an "orphan protein" . . . whose cDNA has been isolated because of its similarity to known proteins." (page 4 of the Office Action) Applicants respectfully remind the Examiner that the specification must be taken as a whole for what it teaches. While the specification does show certain sequence similarities to other proteins (e.g., Fig. 2B), this is far from the extent of characterization of biological significance of R0101 provided. For example, R0101 is shown to be overexpressed in cancer tissue as compared to normal tissue (Fig. 5). R0101 is localized in the nucleus (Fig. 4). R0101 binds PCNA (Figs. 6, 7 and 8;), a well-known cell cycle modulating protein (*see*, e.g., Kelman, Oncogene 14:629-640 (1997), submitted as Exhibit A with Applicants' response filed 9/10/01) and such binding is dependent on an identified region of the r0101 protein (Figs. 7 and 8). And, R0101 competes for binding to PCNA with p21 (Figs. 6 and 8), also a well known cell cycle modulating protein whose cell cycle modulating activity is known to involve interacting with PCNA (*see*, e.g., Kelman, page 637, first full paragraph). Applicants reiterate that the Kelman reference is only provided as an example of what was of general knowledge in the art.

Unfortunately, the above facts are in direct contradiction to statements made in the Office Action. For example, "neither the specification nor the art of record identifies even a single disease or disorder that has been shown to be associated with cell cycle protein R0101". (page 5) In fact, the present specification shows to one of ordinary skill in the art that R0101 is associated with breast cancer, uterine cancer, cervical cancer, brain cancer, kidney cancer, liver cancer, lung cancer, esophageal cancer and rectal cancer (Fig. 5). The Office Action also asserts that ". . . the cell cycle protein

R0101 of the instant invention has not been shown to be associated with a particular physiological process that an artisan would wish to manipulate for assaying bioactive agents for identification of compounds which bind thereto." (page 6) In fact, overexpression of R0101 in the various cancers described above make this an obvious target for identifying agents that bind thereto for diagnostic purposes. R0101's interference with the known binding of PCNA and p21 provides another obvious reason for the presently claimed screens. Furthermore, the overexpression in cancers, localization to the nucleus and interaction with PCNA and p21 disclosed in the present specification provides ample suggestion to the skilled artisan that R0101 is directly involved cell cycle regulation. Applicants submit that the present specification should be considered as a whole with regard to the utility of the claimed invention, as further described below.

The Applicants assert that the present invention, methods for screening for a bioactive agent capable of binding to the cell cycle protein R0101, has utility. Applicants bring to the Examiner's attention evidence within the specification that R0101 expression is increased in certain cancers. The claimed screening methods have utility because they make possible the routine identification of bioactive agents that bind to R0101 protein, *i.e.*, for the diagnosis of, or prognostic evaluation of cancer. With this amendment, Applicants include an expert declaration under 37 C.F.R. § 1.132 by Dr. Yasumichi Hitoshi explaining that one of skill in the art would recognize the utility of the invention claimed in the present application. In addition Applicants attach a peer-reviewed publication by the inventors describing the use of R0101 as a prognostic or diagnostic indicator of certain cancers. (Exhibit A, Yu, *et al.*, *Oncogene* 20:484-489 (2001)).

A. Introduction

According to the MPEP, in order to assess utility, the Examiner should review the specification to determine if there are any statements asserting that the claimed invention is useful for any particular purpose. An invention has utility if the

utility is specific, substantial and credible. A utility is specific if it is specific to the subject matter claimed. A utility is substantial if it has a real-world use. A utility is credible if it would be believable to one of skill in the art. In most cases, an applicant's assertion of utility creates a presumption of utility that is sufficient to satisfy the utility requirement of 35 U.S.C. § 101.

With regard to utility of inventions with pharmacological utility, ". . . a disclosure that identifies a particular biological activity of a compound and explains how that activity can be utilized in a particular therapeutic application of the compound does contain an assertion of specific and substantial utility for the invention." MPEP 2107.02IIA. In addition, ". . .evidence of pharmacological or other biological activity of a compound will be relevant to an asserted therapeutic use if there is a reasonable correlation between the activity in question and the asserted utility." MPEP 2107.03I, *citing Cross v. Iizuka*, 753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985). For molecules that have a demonstrated utility for diagnosis or prognosis of a particular disease, methods to identify materials useful to diagnose the disease, (*e.g.*, materials that bind to the molecule), also have utility. See *e.g.*, Revised Interim Utility Guideline Training Materials, Example 12, page 69-70.

A *prima facie* showing of lack of utility by the Examiner must establish that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the applicant would be specific and substantial. The present application claims methods for screening for a bioactive agent capable of binding to the cell cycle protein R0101. After reading the application, the skilled practitioner would appreciate that R0101 modulates known cell cycle proteins and has increased expression in certain cancers. In addition, the skilled practitioner would (1) know how to routinely identify bioactive agents that bind to R0101 using the claimed methods, and (2) understand that bioactive agents that bind to R0101 are useful for diagnostic and prognostic tests for certain cancers.

B. Examiner's rejections

In the December 18, 2002 Office Action, the Examiner alleges that the instant specification fails to describe the practical utility of the claimed invention. According to the Examiner, there is no evidence showing that the R0101 protein has a biological role or what that role might be. Specifically, the Examiner alleges that neither the specification nor the art of record shows that cell cycle protein R101 is associated with a single disease or disorder. In addition, the Examiner alleges that Applicants have not presented evidence of natural ligands or biological significance of the R0101 protein and therefore there is no patentable use for the protein.

Applicants respectfully traverse. The specification provides both an association between R0101 and cancer and natural ligand for the protein. In addition, In a Declaration under 37 C.F.R. § 1.132, submitted herewith, Dr. Yasumichi Hitoshi explains that the R0101 protein has a physiological role and function, and is overexpressed and therefore, associated with specific cancers. The R0101 protein binds to the PCNA protein, which has a well-established function in DNA synthesis. The R0101 protein is also overexpressed in specific cancers (see attached declaration and Figure 5 of the specification). Furthermore, Dr. Hitoshi explains that because the R0101 protein has the asserted association with specific cancers, methods to identify bioactive agents that bind to R0101 are useful. According to Dr. Hitoshi, bioactive agents that bind to R0101 are useful for diagnosis and prognostic evaluation of specific cancers.

Applicants, therefore, submit that the methods have a specific, substantial and credible utility.

C. R0101 is a cell cycle protein that has increased expression in certain cancers and is associated with certain cancers.

In the Office Action the Examiner alleges that neither the specification nor the art of record shows that cell cycle protein R0101 is associated with a single disease or disorder. Applicants respectfully traverse the allegation and assert that the specification as filed discloses an association between cell cycle protein R0101 and the disease cancer.

Figure 5 of the application shows that R0101 exhibits elevated expression in certain cancers relative to non-cancer cells from the same tissues, *e.g.*, esophageal cancer, breast cancer, uterine cancer, cervical cancer, brain cancer, kidney cancer, and lung cancer. The attached declaration of Dr. Hitoshi demonstrates that the skilled practitioner, after reading the present specification, including Figure 5, would believe increased levels of R0101 are associated with certain cancers, *e.g.*, esophageal cancer, breast cancer, uterine cancer, cervical cancer, brain cancer, kidney cancer, and lung cancer. In addition Applicants attach a peer-reviewed publication by the inventors describing the use of R0101 as a prognostic or diagnostic indicator of certain cancers. (Exhibit A, Yu, *et al.*, *Oncogene* 20:484-489 (2001)).

D. The physiological role of R101 is to bind to PCNA protein.

In the Office Action the Examiner alleges that Applicants have not presented evidence of natural ligands of R0101. Applicants respectfully traverse the allegation and assert that the specification as filed discloses that the PCNA protein, which has a well-known role in DNA synthesis, is a ligand for cell cycle protein R0101. Figure 6 shows that R0101 binds to PCNA in cells and moreover, that R0101 competes with p21 for binding to PCNA. P21 is another protein with a recognized role in the cell cycle and an association with certain cancers. R0101 binding to PCNA occurs through a conserved PCNA binding domain. (See, *e.g.*, figure 2b, p15PAF and R0101 are used interchangeably and refer to the same protein.) In addition, the R0101 binding to PCNA is specific and can be eliminated by specific mutations of the R0101 protein. (See, *e.g.*, Figure 7.) The attached declaration of Dr. Hitoshi demonstrates that the skilled practitioner, after reading the present specification, including Figures 2, 6, and 7, would believe that cell cycle protein R0101 binds to PCNA, a known DNA synthesis protein.

E. Because cell cycle protein R0101 has utilty, methods to identify bioactive agent that bind to R0101 also have utility.

Because of the overexpression of R0101 in certain cancers, one of skill in the art would recognize that bioactive agents that bind to R0101 are useful. For example, one of skill in the art would expect that bioactive agents that bind to a protein known to be overexpressed in certain cancers, as is R0101, would be useful as an indicator of the level of the protein and therefore as a diagnostic or prognostic indicator of those cancers. The declaration by Dr. Yasumichi Hitoshi explains that one of skill in the art would recognize that bioactive agents that bind to R0101 are useful as a diagnostic or prognostic indicator of certain cancers.

F. After reading the present application, the skilled practitioner would know how to identify bioactive agents that bind to R0101.

Methods to determine molecules that bind to a protein are known to those of skill in the art. Applicants have isolated a nucleic acid that encodes the R0101 protein and provide both the nucleic acid (*i.e.*, SEQ ID NO:1) and the encoded amino acid (*i.e.*, SEQ ID NO:2.) Applicants also provide description of bioactive agents at page 23, line 33 through page 27, line 26. Applicants further provide methods determine binding to R0101 and to screen for bioactive agents that bind to R0101 at page 27, line 31 through page 31, line 32.

G. Identification of bioactive agents that bind to R0101 is useful for modulating cell proliferation and for identifying new targets for diagnosis and treatment of cancer.

There are many instances where binding of an agent to a particular protein is useful for diagnosis, determination of prognosis, or treatment of cancer even though the protein itself may not cause cancer. Bioactive agents that bind to proteins that are overexpressed in certain cancers can be used to diagnose the cancer or provide prognostic information about the disease, without a direct connection to the cause of the disease.

For example, p21, a tumor suppressor, is overexpressed in some esophageal cancers and serves as a prognostic indicator of increased patient survival. (See Natsugoe *et al.*, *Clinical Cancer Research*, 5:2445-2449 (September, 1999), attached as Exhibit B). Also, prostate specific antigen (PSA) serves a as useful diagnostic indicator for prostate cancer, even though a direct causal relationship between expression of the protein and the disease ha not been shown. Thus, providing a prognostic or diagnostic test for a particular cancer is useful, even if the original cause of the cancer is unrelated to the protein targeted for diagnostic or prognostic information. Similarly, it is perfectly reasonable to expect that the identification of bioactive agents that bind to R0101, a cell cycle protein that is highly expressed in some cancers, is an appropriate strategy to identify specific diagnostic or prognostic tools for certain cancers.

H. The demonstration that R0101 is overexpressed in certain cancers, coupled with methods for identifying bioactive agents that bind to R0101 and the level of skill in the art is sufficient to provide specific, substantial and credible utility for the claimed methods.

Applicants maintain that the demonstration that the R0101 is overexpressed in certain cancers, coupled with the methods disclosed in the specification and the level of skill in the art of ion channels, is sufficient to demonstrate specific, substantial and credible utility.

Specific utility

Applicants assert that the present invention has a specific utility. Specific utility is defined by the MPEP as a utility that is specific to the subject matter claimed. The MPEP explains that applications show sufficient specific utility when applicants disclose a "specific biological activity" and reasonably correlate that activity to a "disease condition." MPEP 2107.01, 2107.02. In this application, Applicant disclose a "disease condition", *i.e.*, esophageal cancer, breast cancer, uterine cancer, cervical cancer, brain cancer, kidney cancer, and lung cancer, that correlate with a increased expression of R0101. This application also demonstrates that R0101 binds to PCNA, a known cancer

associated protein. This application provides methods of identifying bioactive agents that bind to R0101 for use as a diagnostic or prognostic indicator of the disease. Applicants therefore submit that the present invention has a specific utility, *e.g.*, identification of bioactive agents that bind to R0101 protein for diagnosis or prognostic evaluation of cancer.

Substantial utility

Applicants also assert that the present invention has a substantial or "real world" use. This application provides nucleic acids that encode R0101 protein. The application also demonstrates that R0101 is overexpressed in specific cancers. This application therefore has real world use in the cancer diagnosis and evaluation of prognosis. Throughout the specification, Applicants teach how to identify bioactive agents that bind to R0101 and how to use the bioactive agents. Applicants therefore submit that the present invention has a substantial utility, *e.g.*, the identification of bioactive agents that bind to R0101, useful for diagnostic or prognostic testing of specific cancers.

Credible utility

Finally, Applicants assert that the present invention has a credible utility. According to the MPEP, when an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office Personnel as being "wrong," even when there is reason to believe that the assertion is not entirely accurate. Rather Office Personnel must determine if the assertion of utility is credible, (*i.e.*, whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of the evidence and reasoning provided) MPEP 2107.02 III B. Applicants submit that one of skill in the art after reading this application would (a) know how to identify bioactive agents that bind to R0101 (b) know how to use those bioactive agents that bind to R0101 for diagnostic or prognostic evaluation of specific cancers.

Accordingly, The Applicant respectfully requests that the utility rejection under 35 U.S.C. § 101 be withdrawn.

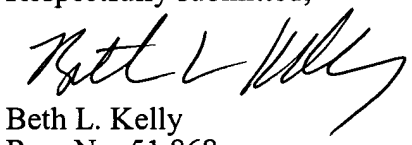
V. Rejection Under 35 U.S.C. §112, First Paragraph, Enablement

Claims 15 and 16 are rejected as not being enabled since the invention is allegedly not supported by either a clear asserted utility or a well established utility. Applicants respectfully traverse the rejection. As described above, the invention claimed in the present application is supported by a specific, substantial, and credible utility. The Office Action also asserts that there is no known activity corresponding with R0101. Applicants traverse the rejection and reiterate that the specification provides an activity for R0101 in binding to PCNA. In addition, the application discloses an association between R0101 and specific cancers. Finally, the specification provides methods of identifying the bioactive agents that bind to R0101 and methods of using those agents. (See, *e.g.*, specification at page 27, line 31 through page 31, line 32, page 40 line 19 through page 53, line 23, and particularly page 41, lines 21-28. Accordingly, Applicants request that the rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is urged. If the Examiner believes a telephone conference would aid in the prosecution of this case in any way, please call the undersigned at 415-576-0200.

Respectfully submitted,



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p15^{PAF}, a novel PCNA associated factor with increased expression in tumor tissues

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Proliferating cell nuclear antigen (PCNA) is an essential protein in both DNA replication and DNA damage repair. A novel 15 kD protein, p15^{PAF}, was identified as a *P*CNA-associated *f*actor in a yeast two-hybrid screen using PCNA as the bait. p15^{PAF} is localized primarily in the nucleus. p15^{PAF} shares the conserved PCNA binding motif with several other PCNA binding proteins including CDK inhibitor p21. Overexpression of p15^{PAF} competes with p21-PCNA binding. Mutation of this motif in p15^{PAF} abolished its PCNA-binding activity. Notably, p15^{PAF} expression in several types of tumor tissues was significantly increased, especially in esophageal tumors. Like PCNA, p15^{PAF} may possess prognostic significance in a broad array of human cancers. *Oncogene* (2001) 20, 484-489.

Keywords: PCNA; p21; tumor; PIP box

Introduction

The proliferating cell nuclear antigen, PCNA, is an auxiliary factor for DNA polymerase processivity. PCNA interacts with numerous DNA replication/repair enzymes, such as DNA polymerase delta and epsilon, replication factor C (RF-C p140), DNA ligase I, replication endonuclease FEN-1, Uracil-DNA glycosylase 2, MLH1, MSH2 and the DNA repair endonuclease, XPG (Chuang *et al.*, 1997; Loor *et al.*, 1997; Umar *et al.*, 1996). Most of these enzymes do not recognize DNA sequences with high specificity. PCNA binds to double strand DNA as a homotrimer and serves as a platform to tether polymerases to the DNA template during DNA synthesis (Kelman and Hurwitz, 1998). PCNA has been shown to interact with DNA (cytosine-5) methyltransferase (MCMT) in post-replication DNA synthesis. MCMT methylation of newly synthesized DNA regulates chromatin organization and gene expression.

Several non-enzymatic cell cycle regulators, such as CDK inhibitors p21$^{CIP/WAF1}$ (Xiong *et al.*, 1992; Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994), p57^{Kip2} (Watanabe *et al.*, 1998), and Gadd45 (Smith *et al.*, 1994; Hall *et al.*, 1995; Sanchez and Elledge, 1995), also bind to PCNA. p21 and p57 both contain two separate binding activities: a CDK-cyclin binding domain at the N-terminus and a C-terminally located PCNA binding site (Watanabe *et al.*, 1998; Luo *et al.*, 1995; Nakanishi *et al.*, 1995), both of which are capable of inhibiting cell cycle progression. Mutagenesis and p21-PCNA crystal structure analysis have identified a short amino acid motif (^{144}QTSMTDFY^{151} in human p21 and ^{268}SGPLISDFF^{276} in human p57) critical for binding to PCNA. In particular, the hydrophobic Met/Leu and Phe residue (underlined), are required for p21 and p57 to bind PCNA at high affinities (Watanabe *et al.*, 1998; Warbrick *et al.*, 1995; Gulbis *et al.*, 1996). In addition to these two CDK inhibitors, a similar conserved PCNA binding motif (also named PIP-box) containing QXXL/I/MXXF/Y (Warbrick, 1998), is also found in several other PCNA interacting proteins, including xeroderma pigmentosum group G (XPG; Ludwig *et al.*, 1997), flap endonuclease (FEN-1; Li *et al.*, 1995) and DNA-(cytosine-5) methyltransferase (MCMT; Chuang *et al.*, 1997). The p21-PCNA association does not affect the overall structure of PCNA or the PCNA-DNA association (Gulbis *et al.*, 1996). Instead, p21 is capable of competing with these PCNA-binding proteins, potentially preventing PCNA from binding to DNA polymerase and other replication factors. In this study, we identified a novel 15 kD PCNA-associated factor, p15^{PAF}. p15^{PAF} contains the conserved PCNA binding motif in which a mutation disrupts PAF-PCNA binding. Unlike p21 and p57, overexpression of p15^{PAF} does not inhibit cell cycle progression. Of potential significance, the expression of p15^{PAF} is substantially elevated in several types of tumors. We suggest that p15^{PAF} may be a new member of PCNA associated cell proliferation family of regulators.

Results

Identification of p15^{PAF}

A large number of proteins have been identified through binding to PCNA. To clone less abundant

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PCNA associated proteins, we constructed a yeast two-hybrid cDNA library from mixed human tissues of adult whole brain, fetal whole brain, thalamus, and hippocampus at 1:1:1:1 ratio instead of using only whole brain mRNA. This library contains 36 million independent clones. Twenty million transformants were screened. A total of 73 positive clones were identified, including three known PCNA binding proteins (p21, FEN-1, and Uracil-DNA glycosylase 2), validating the authenticity and efficiency of the screen. Five of the 73 PCNA-interacting clones contained cDNA inserts corresponding to the expressed sequence tag (EST) clone KIAA0101 deposited in GenBank (Nagase *et al.*, 1995) (Figure 1a). These five positive clones all contain the PCNA binding motif and encode the entire coding region of KIAA0101. KIAA0101 (D14657) was conceptually translated but is functionally uncharacterized. Due to its PCNA-binding activity, we have therefore named KIAA0101 as PAF (PCNA associated factor). Notably, PAF contains sequence closely related to the PCNA-binding motif present in other PCNA-interacting proteins (Warbrick, 1998) (Figure 1b). In particular, three highly conserved residues, Gln, Met/Ile/Leu and Phe, are all conserved in PAF, suggesting that PAF may bind to PCNA in a similar manner as other PCNA binding proteins.

p15PAF binds to PCNA in mammalian cells

Conceptual translation of PAF predicts a 15 kD protein (p15PAF), which was confirmed by transient transfection and immunoprecipitation (Figure 2). To confirm the binding of p15PAF with PCNA in mammalian cells, HA-tagged p15PAF was recloned from the positive yeast-two-hybrid clone into a CMV promoter expression vector (pYCI) by PCR and was transfected into 293 cells. Figure 2 shows HA-tagged p15PAF co-immunoprecipitated with endogenous PCNA (Figure 2a,b). Mutation of two conserved residues in the putative PCNA binding motif (I65A, F68A) of the PIP-box, completely disrupted the binding of p15PAF to PCNA (Figure 2c, lane 3). The lower panel of Figure 2c shows that both the wild type and the mutant p15PAF were expressed at similar protein levels. This result demonstrated that the PIP-box is required for p15PAF binding to PCNA.

p15PAF competes with p21 for binding to PCNA

p21 has been shown to compete with DNA polymerase and FEN-1 for binding to PCNA. Since both p15PAF and p21 share the same PCNA-binding motif, we tested whether p15PAF can compete with p21 for binding to PCNA. HA-tagged p15PAF was co-transfected with Flag-tagged p21 into 293 cells, and p15PAF binding to PCNA was diminished in the presence of increasing concentration of p21 (Figure 2b, upper panels). When Flag-tagged p21 was co-transfected with GFP or HA-tagged p15PAF into 293 cells, p21's binding to PCNA was also inhibited by increasing the amount of p15PAF (Figure 2b, lower panels). Results from HA-tagged p15PAF co-transfection are not shown. Since only very high levels of p15PAF (6:1 ratio) are able to compete with p21 for PCNA association, it is speculated that the affinity of p15PAF to PCNA may be weaker than that p21, although endogenous levels of p21 and p15PAF were not measured in transfection experiments.

Tissue-specific expression and nuclear localization of PAF

PAF gene was expressed as a 1.1 kb message that accumulated in liver, pancreas and placenta at a high levels (Figure 3). PAF expression was not detected in heart or whole adult brain, despite the initial isolation from a brain cDNA library. To determine the subcellular localization, we fused full length PAF to the C-terminal of GFP and expressed PAF-GFP protein in Hela cells by transient transfection. As shown in Figure 4, the majority of green fluorescence was observed in the nucleus, although some cytoplasmic distribution can also be seen. Hence, PAF is mainly localized to the nucleus.

p15PAF expression is increased in tumor tissues

PCNA expression has been correlated to tumor progression and is commonly used as a molecular marker for detecting hyperplastic cell growth. Increased p21 expression has also been seen in several types of malignant tumors (Buckelandt *et al.*, 1999; Barboule *et al.*, 1998), and a higher level of p21 has been correlated with higher 5-year survival rate (Kuwahara *et al.*, 1999; Natsugoe *et al.*, 1999; Ropponen *et al.*, 1999). To evaluate p15PAF's potential function in tumor development, p15PAF expression level was measured using tumor blots (Invitrogen). As shown in Figure 5, the p15PAF mRNA level was significantly increased in esophageal, breast, uterine, cervix, brain, kidney and lung tumors. This increased mRNA level is especially dramatic in esophageal tumor (>10-fold). Northern analysis also verified that in normal whole adult brain tissue, little or no p15PAF was detected. Little or an undetectable change of p15PAF expression was observed in colorectal cancer or pancreas tumor.

Discussion

Both PCNA and p21 have been shown to interact with a large number of cellular proteins involved in DNA replication/repair and cell cycle control. Most of the non-enzymatic PCNA binding proteins identified, such as p21 and p57, inhibit DNA synthesis and cell cycle progression. Since p15PAF is able to compete with p21 for binding to PCNA, it is plausible to speculate that over-expression of p15PAF in tumor tissues may be advantageous for tumor cell proliferation. However, co-transfection of p15PAF with p21 expression plasmid into Saos-2 and 293 cells did not show a significant change in p21 induced cell cycle arrest. In addition, PCNA has more than one p21/p15PAF binding site and may bind to both p15PAF and p21

a

b

MVRTKADSVPGTYRKVVAARAPRKVLGSSTSATNSTSVSSRKAENKYAGG 50

NPVCVRPTPKWQKGIGEFTRLSPKDSEKENQIPEEAGSSGLGKAKRKACP 100

LQPDHTNDEKE 111

c

p15PAF	(62-69)	QKGIGEFF
P21	(142-149)	QTSMTDFY
P57	(269-276)	GPLISDFF
XPG	(991-999)	QLRIDSFF
FEN1	(333-340)	QGRLDDFF
MCMT	(164-171)	QTTITSHF

Figure 1 p15PAF was identified in a yeast two-hybrid screen using PCNA as the bait. (a) Cloning of p15PAF by binding to PCNA. Yeast Y190 cells were simultaneously transformed with a plasmid expressing a GAL4bd fusion protein and a plasmid expressing a GAL4ad fusion protein as indicated. Cells were streaked on non-selective medium with histidine (−Leu, −Trp), selective medium without histidine (−Leu, −Trp, −His) and selective medium without histidine but containing 5 mM 3-amino-1,2,3-triazole (−Leu, −Trp, −His, 3-AT). Staining for β-galactosidase expression, activated from an independent GAL4 responsive promoter, is shown lower right panel (denoted by β-gal). The C-terminal domain of p21 possesses a trans-activating activity (self-activation) when expressed as fusion protein with the GAL4 DNA binding domain. (b) Amino acid sequence of human PAF/KIAA0101. The conserved PCNA binding motif is underlined, also known as PIP-box. (c) Comparison of PCNA binding motif. The number in parenthesis indicate the position of PCNA-binding motif in the respective proteins

at the same time. Without knowing the relative endogenous protein level of p15PAF and p21, it is difficult to determine the relative relationship between p15PAF and p21. Under physiological conditions, p15PAF may not be able to compete with p21 for PCNA binding. p21 binds to PCNA to disrupt DNA replication/repair machinery and to inhibit cell proliferation. However, in transient transfection experiments using Hela, Saos-2, and 293 cells, we were not able to detect any cell cycle inhibition by the overexpression of p15PAF (data not shown). An *in vitro* DNA synthesis assay may be needed to reveal the detailed mechanism of p15PAF function.

Since the mRNA level of p15PAF is dramatically increased in many types of tumors, we amplified (by PCR) and sequenced p15PAF from kidney, liver, lung and esophageal tumors. No mutation was found in p15PAF

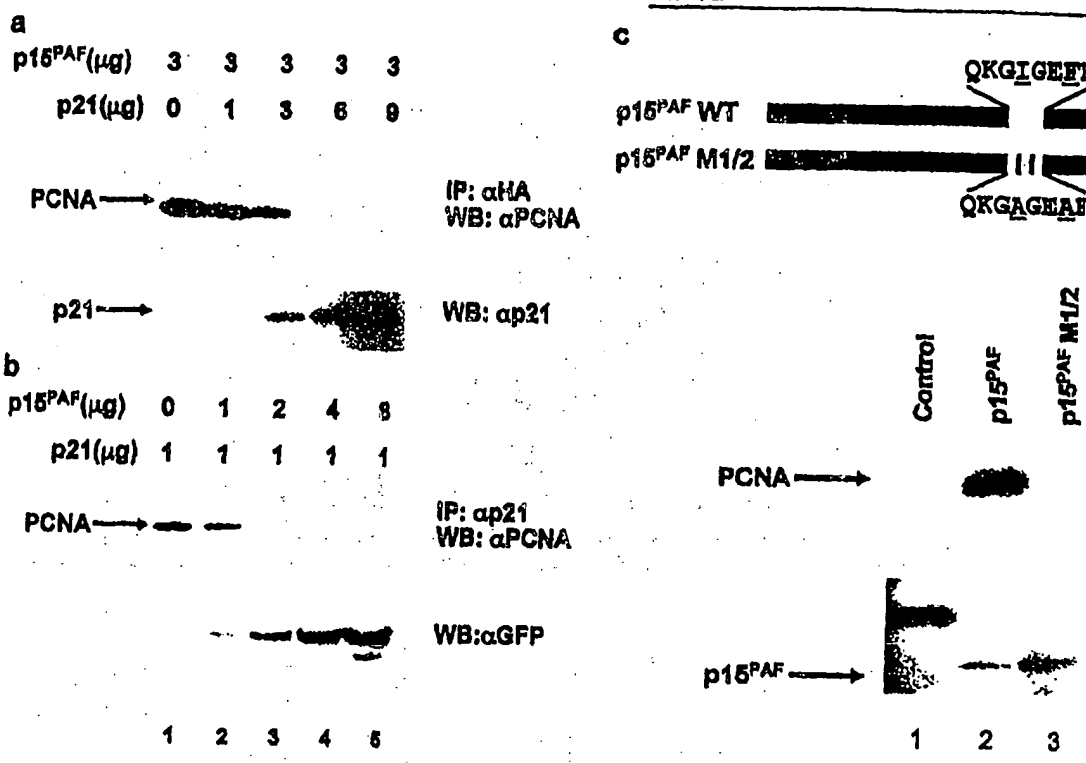

Figure 2 p15PAF competes with p21 for binding to PCNA in mammalian cells. (a) p21 competes with p15PAF for binding to endogenous PCNA. 3 μg of HA-tagged p15PAF expression plasmid (pYC1) was co-transfected with 0–9 μg of p21 expression plasmid into 293 cells (Phoenix A cells). Cell lysate was immunoprecipitated by anti-HA antibody and subsequently blotted with anti-PCNA antibody. The amount of p21 protein in a control experiment is shown in the lower part of the panel. (b) p15PAF competes with p21 for binding to endogenous PCNA. 1 μg of p21 expression plasmid was co-transfected with 0–8 μg GFP-tagged p15PAF expression plasmid. Cell lysate was immunoprecipitated by anti-p21 antibody and subsequently blotted with anti-PCNA antibody. In the control experiment shown in the lower part of the panel, the amount of GFP-tagged p15PAF is shown by blotting with an anti-GFP antibody. (c) Mutation of PIP-box disrupts binding of p15PAF to PCNA. 3 μg of HA-tagged p15PAF wt/mutant plasmid was transfected into 293 cells (Phoenix A cells). Cell lysate was immunoprecipitated with anti-HA antibody and blotted with anti-PCNA antibody (upper panel). Lower panel shows protein amount of p15PAF. Control lane is a non-specific HA-tagged protein

(data not shown). This result excludes the possibility that a dysfunctional copy of p15PAF is over-expressed in tumor tissues. Increased expression of p15PAF in many tumor tissues is not surprising. Expression of PCNA and PCNA-associated proteins such as p21 is also increased in several types of cancers including ovarian cancer and breast cancer. The inhibitory effect of p21 may be overcome by coordinated accumulation of PCNA, cyclinD1 and CDKs (Russell *et al*., 1999). Similarly, the impact of increased expression of p15PAF in tumor tissues may be neutralized by elevated expression of its binding partners, such as PCNA. However, elevated levels of p15PAF in tumor tissues may be a useful prognostic parameter for certain types of cancer. In esophageal squamous cell carcinoma patients, for example, the 5-year survival rate of p21 positive patients is better than that of p21 negative patients. In other types of cancer, p21's value as an independent prognostic marker is not conclusive. Since p15PAF expression in esophageal tumor is dramatically elevated compared with normal tissue, the possibility exists that p15PAF levels could be used to predict clinical prognosis for esophageal cancer patients.

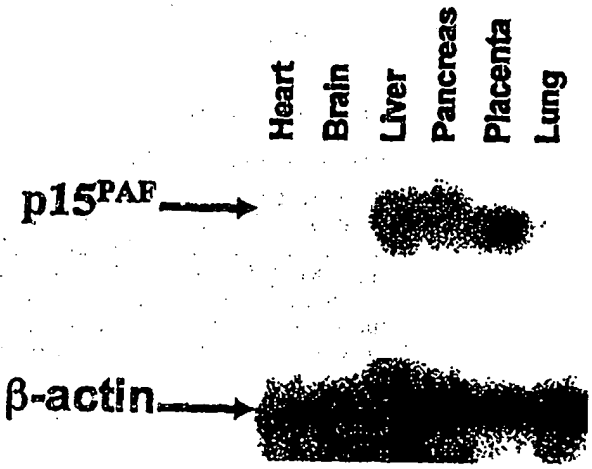

Figure 3 Northern blot of p15PAF tissue expression pattern. Northern blot was purchased from Invitrogen (D1801-08). The band of p15PAF is indicated by arrow. β-actin is used as control. The DNA probe of p15PAF was ^{32}P labeled using an Ambion kit (#1455). Kodak X-ray film was exposed for 24 h after hybridization

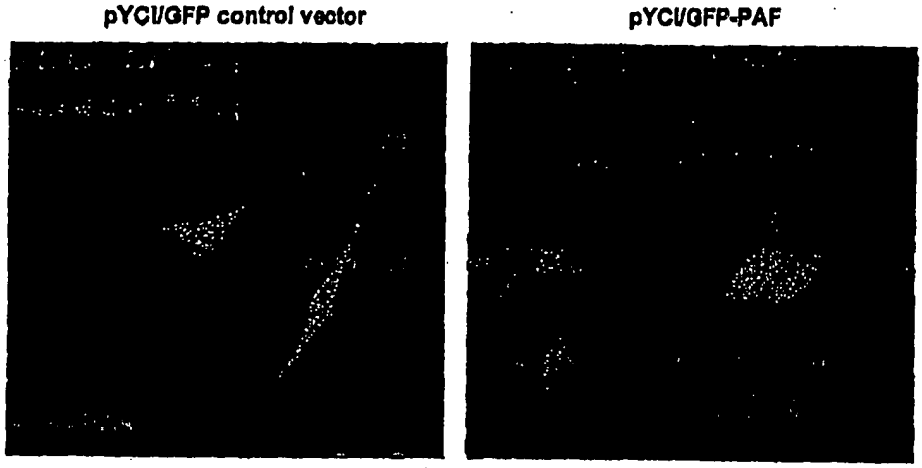

Figure 4 p15PAF is localized in the nucleus. GFP-tagged control vector and PAF–GFP were transiently transfected into Hela cells. Twenty-four hours after transfection, fluorescence was recorded using a Nikon TE300 fluorescence microscope

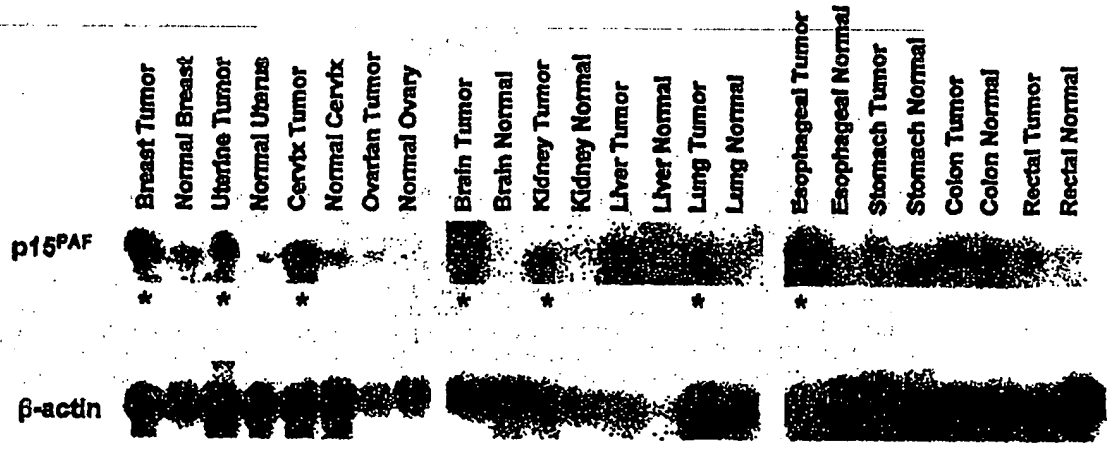

Figure 5 p15PAF is highly expressed in some tumor tissues. Human tumor tissue panel blots were purchased from Invitrogen (D3100-01, D3200-01, D3400-01). β-actin probe was used as control. ^{32}P-labeled p15PAF probe was used and the X-ray film was exposed for 48 h. Tumor tissues with elevated level of p15PAF, such as breast, uterine tissue, cervix, brain, kidney, lung and esophageal tumors are labeled by * underneath (upper panel). The p15PAF band in brain tumor tissue was diffuse in two different tumor blots

Materials and methods

Yeast two-hybrid screening

For yeast two-hybrid screening (Fields and Song, 1989), full length PCNA was used as bait to screen a human brain cDNA library constructed from mixed mRNA of whole adult brain, whole fetal brain, hippocampus, and thalamus. Oligo-dT primer was used to make a unidirectional cDNA library and was ligated into *XhoI–EcoRI* sites of the pACT2 vector. Random hexamers were used to construct a second bi-directional library and was ligated into the *EcoRI* site of pACT2. The combined cDNA library represents 36 million independent clones. Yeast strain Y190 was used and 20 million transformants were screened on SD-LWH+3AT (45 mM) plates from a single round of screening.

Transfection and immunoprecipitation

The Ca^{2+} phosphate method was used in all transfection experiments. 2×10^6 Phoenix-A (293 T) cells were harvested 24 h after the transfection of HA-p15PAF (5 μg) and lysed in 0.5 ml lysis buffer (50 mM HEPES [7.6], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA). The immunoprecipitated proteins were prepared by incubating the anti-HA monoclonal Ab with the cell lysates. Volumes were adjusted by normalization with expression of Flag-tagged or HA-tagged proteins analysed by Western blot analysis (protein levels before adjustment for IP shown in middle and bottom panels of Figure 2). The anti-HA immunoprecipitated samples were then washed three times with high stringency lysis buffer (1% NP-40 and 1 M NaCl). For each immunoprecipitation following normalization, aliquots of the lysates were incubated with a 1:1 slurry of anti-HA conjugated Sepharose (BAbCO). The Sepharose beads were washed once with 1 ml lysis buffer and three times with high stringency lysis buffer (1 M NaCl, 1% NP-40). The immunoprecipitated proteins or cell lysates were fractionated on a 4–20% gradient SDS–PAGE gel (Novex).

Cell cycle assay

A549 cells were infected with vector or p15PAF retrovirus supernatant which were collected from transfected Phoenix A

retroviral packaging cells. The top 10% of GFP positive A549 cells 48 h post infection were isolated and cell cycle analysis performed with hypotonic PI solution staining by FCS 72 h post infection. The FCS data was analysed by ModFit LT cell cycle analysis software (Verity Software House, ME, USA).

Fluorescence

GFP-fused p15PAF was transfected into 293 (Phoenix) cells. A Nikon TE-300 fluorescence microscope was used to monitor localization in the transfected cells. The UV excitation wavelength is between 420 and 490 nm and the GFP emission is monitored at 520 nm.

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Expression of $p21^{WAF1/Cip1}$ in the p53-dependent Pathway Is Related to Prognosis in Patients with Advanced Esophageal Carcinoma[1]

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ABSTRACT

The proteins p53 and p21 are important components that regulate G_1-S transition through the cell cycle. We immunohistochemically investigated p53 and p21 expression in 111 patients with esophageal squamous cell carcinoma. We also evaluated whether the expression of either of these proteins is a prognostic factor according to the p53-dependent and -independent pathways. The positive rates of p53 and p21 expression were 42.8 and 43.2%, respectively. Clinicopathological findings according to p53 and p21 expression did not differ significantly. The 5-year-survival rates between p21 positive and negative expression did not differ significantly in the p53-positive group. In the p53-negative group, the 5-year-survival rate of patients with p21-positive expression was 22.9%, which was significantly better than that of patients with p21-negative expression (12.7%; $P < 0.05$). Multivariate analysis revealed that p21 expression in the p53-dependent pathway was an independent prognostic factor. Accordingly, the prognostic values of p21 expression between the p53-dependent and -independent pathways differed. Examination of p21-positive expression in the p53-dependent pathway will help to estimate the favorable prognosis of patients with advanced esophageal carcinoma.

INTRODUCTION

The tumor suppresser gene *p53* is located on the short arm of chromosome 17. The product of wild-type p53 prevents uncontrolled cellular proliferation after DNA damage via a G_1 arrest checkpoint (1, 2). Mutation of the *p53* gene is one of the most frequent genetic lesions associated with cancer, including esophageal cancer (3, 4). The p21 protein, which is encoded by the *WAF1/Cip1* gene, is a downstream target effector of wild-type p53, which transcriptionally activates p21 (5, 6). The relationship between p21 expression and tumors in the gastrointestinal tract has been reported. Furthermore, p21 expression is an important prognostic factor in various organs (7–10). The p53-dependent expression of p21 plays a central role in cell growth regulation and apoptosis. p21 can be induced in a p53-independent manner (11, 12). Understanding p53 and p21 protein expression is important for gene function in malignant tumors. Both p53-dependent and -independent pathways must be examined when considering p21 expression.

Although *p53* gene mutation and p53 protein accumulation are common in esophageal cancer, their clinical significance is controversial (13, 14). In this study, we examined p53 and $p21^{WAF1/Cip1}$ (p21) expression in advanced esophageal squamous cell carcinoma according to these protein accumulation detected by immunohistochemical methods. The aim of this study was to investigate the relationship between p53 and/or p21 expression and to evaluate whether their expression is a prognostic factor according to p53-dependent and -independent pathways.

PATIENTS AND METHODS

Patients. One hundred and eleven consecutive patients with advanced carcinoma of the esophagus underwent esophagectomy with lymph node dissection at Kagoshima University Hospital between 1987 and 1991. The ages of 103 male and 8 female patients ranged from 41 to 81 years (mean, 64.0 years), and none of them had received radiation therapy or chemotherapy before surgery. All patients were followed up after discharge by an X-ray examination and studies of tumor markers (squamous cell carcinoma antigen, carcinoembryonic antigen) every 1–3 months, computed tomography every 3–6 months, and ultrasonography every 6 months. Bronchoscopic and endoscopic examinations were performed when necessary. Follow-up data after surgery were obtained from all patients, with a median follow-up period of 28 months (range, 2–135 months).

On the basis of the Tumor-Node-Metastasis classification of the International Union Against Cancer (15), the 111 patients were divided into 18 with T_2 tumors, 66 with T_3 tumors, and 27 with T_4 tumors. Seventeen tumors were located in the upper third of the esophagus, 59 in the middle third, and 37 in the lower third. Pathologically, all of the tumors were squamous cell carcinoma (51 well-differentiated, 42 moderately differentiated, and 18 poorly differentiated). Lymph node metastases were present in 77 of 111 of the patients (69.4%). All of the M1 tumors were due to distant lymph node metastases.

Immunohistochemical Staining. Sections were immunohistochemically stained using avidin-biotin-peroxidase as described. In brief, after deparaffinizing in xylene and dehydrating

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EXHIBIT B

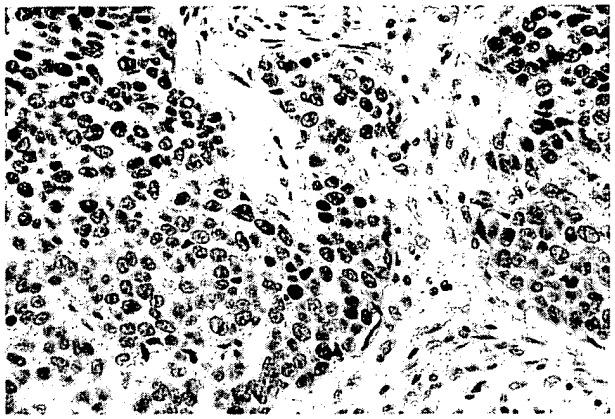

Fig. 1 p21 expression is seen in the nucleus in esophageal cancer cells (×200).

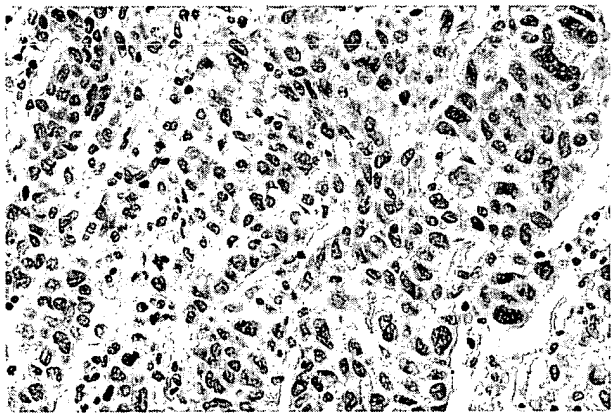

Fig. 2 p21 expression is not seen in the nucleus in esophageal cancer cells (×200).

in ethanol, the sections were heated in citrate buffer (0.01 M, pH 6.5) at 120°C for 10 min to retrieve antigen, then incubated with either the primary monoclonal antibody anti-$p21^{WAF1/Cip1}$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-p53 (Transduction Laboratories, Lexington, KY) overnight at 4°C. The sections were then incubated with biotinylated anti-mouse IgG and avidin-biotin-peroxidase (Vector Laboratories, Burlingame, CA) and visualized using diaminobenzidine tetrahydrochloride. The negative control group contained 1% BSA instead of primary antibody.

The immunohistochemical expression of p53 and p21 was evaluated by independent two observers (Sh. N. and Sa. N.). The five representative fields were examined, and a total of 1000 tumor cells (200 for each field) were counted under the microscope with a high power (×200) objective. A distinct nuclear immunoreaction for p53 and p21 was judged positive. When 10% of the cancer cells were positive for nuclear staining, the specimen was scored as positive (Figs. 1 and 2). The level of wild-type p53 protein is usually undetectable in tissues by immunohistochemical staining because of its short half-time. Because the mutated p53 protein accumulates in the nucleus as a consequence of its binding to other oncogenic proteins, such

Table 1 Relationship between p21 expression and clinicopathological findings

	p21(+) (n = 48, %)	p21(−) (n = 63, %)	P
Age	63.9 ± 9.1	64.1 ± 8.1	NS[a]
Gender (male:female)	46:2	57:6	NS
Tumor location			NS
Upper	7 (14.6)	10 (15.9)	
Middle	23 (47.9)	34 (54.0)	
Lower	18 (37.5)	19 (30.1)	
Tumor depth			NS
T_2	9 (18.8)	9 (14.3)	
T_3	29 (60.4)	37 (58.7)	
T_4	10 (20.8)	17 (27.0)	
Histology			NS
Well	21 (43.8)	30 (47.6)	
Moderate	21 (43.8)	21 (33.3)	
Poor	6 (12.4)	12 (19.1)	
pN			NS
pN_0	15 (31.3)	18 (28.6)	
pN_1	33 (68.7)	45 (71.4)	
pM			NS
pM_0	28 (58.3)	45 (71.4)	
pM_1	20 (41.7)	18 (28.6)	
Stage			NS
II A	13 (27.1)	13 (20.6)	
II B	3 (6.3)	4 (6.4)	
III	12 (25.0)	24 (38.1)	
IV	20 (41.6)	22 (34.9)	
p53 expression			NS
Negative	27 (56.2)	37 (58.7)	
Positive	21 (43.8)	26 (41.3)	

[a] NS, not significant.

binding prolongs its half-life (16). Thus, the p53 protein detected by immunohistochemistry is considered to be the product of a mutated gene for p53. On the other hand, the p21 protein detected by immunohistochemical staining is considered to be a wild-type protein because no mutations in the gene for p21 were detected in a large number of human tumors (17, 18).

Statistical Evaluation. Statistical analysis was performed by the χ^2 test and the Kruskal-Wallis test for group differences. The Kaplan-Meier method was used, and analysis was also evaluated by the log-rank test. Prognostic factors were tested by univariate and multivariate analyses (proportional hazard regression model). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of p53 and p21

The rate of p53 expression was 42.8% (47 of 111) for all patients. The relationship between p53-positive and -negative expression and the clinicopathological findings of age, sex, tumor location, depth of tumor, histology, and pN did not differ significantly. However, the rate of advanced tumors in pM_1 and the stage were significantly higher in p53-positive tumors than in p53-negative tumors. The positive rate of p21 was 43.2% (48 of 111) for all patients. Clinicopathological findings and p21 expression did not significantly differ, and neither did p53 and p21 expression (Table 1).

The tumors were divided according to p53 expression into

Table 2 Relationship between p21 expression and clinicopathological findings according to p53 expression

	p53(+)		p53(−)		
	p21(+) (n = 21, %)	p21(−) (n = 26, %)	p21(+) (n = 27, %)	p21(−) (n = 37, %)	P
Tumor location					NS[a]
Upper	4 (19.0)	5 (19.2)	3 (11.1)	5 (13.5)	
Middle	9 (42.9)	10 (38.5)	14 (51.9)	24 (64.9)	
Lower	8 (38.1)	11 (42.3)	10 (37.0)	8 (21.6)	
Tumor depth					NS
T_2	4 (19.0)	4 (15.4)	5 (18.5)	5 (13.5)	
T_3	11 (52.4)	14 (53.8)	18 (66.7)	23 (62.2)	
T_4	6 (28.6)	8 (30.8)	4 (14.8)	9 (24.3)	
Histology					NS
Well	9 (42.9)	10 (38.5)	12 (44.4)	20 (54.1)	
Moderate	9 (42.9)	9 (34.6)	12 (44.4)	12 (32.4)	
Poor	3 (14.2)	7 (26.9)	3 (11.2)	5 (13.5)	
pN					NS
pN_0	3 (14.3)	9 (34.6)	12 (44.4)	9 (24.3)	
pN_1	18 (85.7)	17 (65.4)	15 (55.6)	28 (75.7)	
pM				[b]	$P < 0.05$
pM_0	8 (38.1)	15 (57.7)	20 (74.1)	26 (70.3)	
pM_1	13 (61.9)	11 (42.3)	7 (25.9)	11 (29.7)	
			[b]		
Stage				[b]	$P < 0.05$
IIA	3 (14.3)	7 (26.9)	10 (37.0)	6 (16.2)	
IIB	2 (9.5)	3 (11.6)	1 (3.7)	1 (2.7)	
III	3 (14.3)	5 (19.2)	9 (33.3)	19 (51.4)	
IV	13 (61.9)	11 (42.3)	7 (26.0)	11 (29.7)	
			[b]		

[a] NS, not significant.
[b] $P < 0.05$.

the p53-positive [p53(+)] and p53-negative [p53(−)] groups. With regard to clinicopathological findings in the p53(+) group, there was no significant relationship between the p21(+) and p21(−) group. Similarly, the relationship among clinicopathological findings in the p53(−) group was not significant, irrespective of p21 expression. The categories of M_1 and stage were more advanced in the p53(+)p21(+) group than in the p53(−)p21(+) or p53(−)p21(−) groups (Table 2).

Clinical Outcome

The Prognosis of p53 Expression or p21 Expression. All patients were followed up, and 8 died of postoperative complications within 30 days, leaving 103 patients for survival analysis. The total number of 5-year survivors was 19, and 15 of these patients are still alive. According to the p53 expression, the 5-year survival rates of the p53(+) and p53(−) patients were 14.9 and 18.8%, respectively (P = 0.19). Concerning the p21 expression, the 5-year survival rates were 22.9% for patients with p21-positive tumors and 12.7% for those with p21-negative tumors (P = 0.08).

The Prognosis of p21 Expression in the p53-dependent or -independent Pathway. In the p53(+) group, the 5-year survival rates of the p21(+) and p21(−) patients were 19.1 and 11.5%, respectively. There was no significant difference between the two groups. On the other hand, in the p53(−) group, the 5-year survival rate of the p21(+) patients (25.9%) was significantly higher than that of the p21(−) patients (13.5%; $P < 0.05$; Fig. 3).

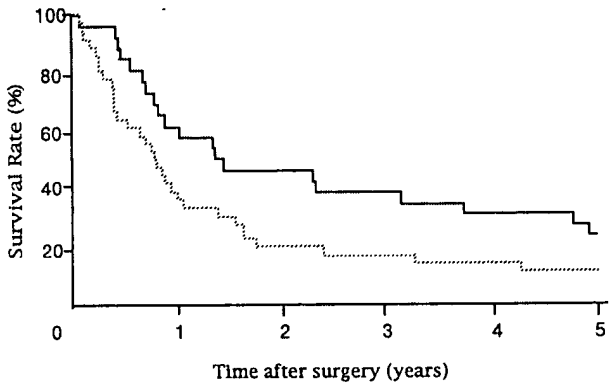

Fig. 3 A comparison of 5-year survival curves in the p53-dependent pathway between patients with p21-positive tumors (*thick lines*) and p21-negative tumors (*broken lines*) is shown. The 5-year survival rate of the p21-positive group is significantly higher than that of the p21-negative group ($P < 0.05$).

Prognostic Significance of p21 Expression in the p53-dependent Pathway. Factors related to prognosis within the p53(−) group were evaluated by univariate and multivariate analysis (Table 3). According to the univariate analysis, age, histology, depth of tumor invasion, lymph node metastasis, lymphatic invasion, venous invasion, and p21 expression were related to prognosis. However, age, stage, and p21 expression

Table 3 Risk factors affecting overall survival rate determined by univariate and multivariate analysis of prognostic factors for the p53-dependent pathway in 103 patients with esophageal carcinoma

Independent factor	*P*		Relative risk	95% CI[a]
	Univariate	Multivariate		
Age	0.0231	0.0194	1.042	1.006–1.079
Stage (II *vs.* III and IV)	0.0020	0.0034	2.986	1.435–6.25
p21 expression (+ *vs.* −)	0.0193	0.0402	1.867	1.029–3.387

[a] CI, confidence interval.

were independent prognostic factors, according to the multivariate regression analysis.

DISCUSSION

The tumor suppressors p53 and p21 are among the most important known gene products involved in cell growth arrest, differentiation, and senescence. The p21 protein is a cyclin-dependent kinase inhibitor that is a downstream effector of p53-dependent cell cycle regulation (19, 20). Although to date, some reports have addressed the relationship between poor prognosis and p53 overexpression in esophageal carcinoma (21, 22), others did not find such correlation (23, 24). Sarbia *et al.* (25) reported recently that p21-positive expression correlates with the poor prognosis of patients with esophageal carcinoma. In the present study, we compared p53 and p21 protein expression by p53-dependent and -independent pathways.

In this series, p21 expression and clinicopathological findings were not correlated. Histologically, p21 plays a role in regulating the cellular differentiation of various tissues. Heterogeneous components were identified in advanced esophageal carcinoma; some areas were well differentiated, and others were moderately or poorly differentiated. However, we did not find a significant difference between p21 expression and histology. Furthermore, p21 and p53 expression did not correlate in the present study. Factors such as transforming growth factor β (26), cyclin D1 (27), and bcl-2 (28) might influence p21 expression. Barboule *et al.* (29) also reported that the absence of p21 expression does not correlate with wild-type p53 in ovarian cancer. However, the p53(+)/p21(+) tumors were more advanced than those tumors of the p53(−) group. Tumors that expressed p21 progressed faster via the p53-independent than the p53-dependent pathway, suggesting that p53 mutant tumors are highly malignant, even in the presence of p21 protein.

Age, histology, depth of tumor invasion, lymph node metastasis, lymphatic invasion, and venous invasion were important prognostic factors among patients with advanced esophageal carcinoma in the present study. Lymph node metastasis or the number of involved nodes are useful prognostic factors (30, 31). In this study, although the 5-year survival rate of p21-positive patients (22.9%) tended to be better than that of p21-negative patients (12.7%), the difference did not reach significance ($P = 0.08$). However, when patients were divided according to p53 expression, *i.e.*, the p53-dependent or p53-independent pathway, the prognosis was better in p21-positive than in p21-negative patients in the p53-dependent group. Furthermore, p21 expression was an independent prognostic factor in the wild-type p53 pathway by multivariate analysis. Sarbia *et al.* (25) reported that the patients with p21 positivity had a poorer prognosis, compared with those with p21 negativity, when p21 expression was categorized into <50% positive cells and ≧50% positive cells. This result was different from ours, which was caused by different criteria of p21 expression. Although the prognosis of patients with advanced esophageal carcinoma remained poor, the examination of the pathway of p53-dependent p21 protein expression is useful for predicting prognosis in addition to the conventional staging system.

We conclude that the prognostic value of p21 expression differed between p53-dependent and -independent pathways. An examination of p53 and p21 expression is useful when estimating the prognosis of patients with advanced esophageal carcinoma.

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Publications

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Patent

1. Toso, a cell-surface specific regulator of Fas-induced apoptosis in T cells
Stanford Docket S98-019